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FORM PTO-1390 (REV. 12-29-99) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 96429/9085

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/463276

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/15387	24 July 1998	26 July 1997

TITLE OF INVENTION: TRANS-SPECIES NUCLEAR TRANSFER

APPLICANT(S) FOR DO/EO/US: Neal L. First, Tanja Dominko and Maissam Mitalipoua

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Ofice (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19(35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

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U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/463276	PCT/US98/15387	96429/9085

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$690.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 670.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	15 - 20 =	0	X **$18.00**	$
Independent claims	3 - 3 =	0	X **$78.00**	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$260.00**	$
TOTAL OF ABOVE CALCULATIONS =				$ 800.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statment must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$ 400.00
SUBTOTAL =				$ 400.00
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$
TOTAL NATIONAL FEE =				$ 400.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$
			Amount to be refunded:	$
			charged:	$

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0842. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pendin g status.

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33,049
REGISTRATION NUMBER

Attorney's Docket No. 96429.9008

Applicant or Patentee: Neal L. First et al.

Serial or Patent No.: via PCT

Filed or Issued: 7-24-1998

For: UNIVERSAL MAMMALIAN CLONING INCUBATOR VEHICLE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled UNIVERSAL MAMMALIAN CLONING INCUBATOR VEHICLE

described in

☐ the specification filed herewith.

☒ application serial no. via PCT, filed 7-24-1998.

☐ patent no. _____, issued _____.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☐ no such person, concern, or organization

☒ persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME: Wisconsin Alumni Research Foundation

ADDRESS: P.O. Box 7365, Madison, WI 53707-7365

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

NAME: _____

ADDRESS: _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

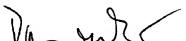
I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Neal L. First
Name of inventor

_____ Date _____
Signature of inventor

Tanja Dominko
Name of inventor


Date 04/29/2000
Signature of inventor

Maissam Mitalipova
Name of inventor

_____ Date _____
Signature of inventor

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Neal L. First
Name of inventor

Neal L. First
Signature of inventor

Date 5-2-00

Tanja Dominko
Name of inventor

Signature of inventor

Date

Maissam Mitalipova
Name of inventor

5/8/00

Signature of inventor

Date

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

NAME: _____

ADDRESS: _____

_____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME: _____

ADDRESS: _____

_____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Bryan Z. Renk

TITLE IN ORGANIZATION Director of Patents and Licensing

ADDRESS OF PERSON SIGNING Wisconsin Alumni Research Foundation

P.O. Box 7365, Madison, WI 53707-7365

SIGNATURE Bryan Z. Renk Date 5/1/00

TRANS-SPECIES NUCLEAR TRANSFER

FIELD OF THE INVENTION

The present invention relates to the field of the cloning of mammals by nuclear transfer, and to a method of producing nuclear transfer embryos from a variety of cell types, including producing embryos using a donor cell from one species and a recipient oocyte from another species. The method may also be used to produce transgenic animals.

BACKGROUND OF THE INVENTION

Cloning by nuclear transfer was originally described in amphibians (for review see Gurdon, 1986 and DiBerardino, 1987). More recently, cloning by nuclear transfer has been described in the following mammalian species: sheep (Willadsen, 1986; Campbell et al., 1996; and Wilmut et al., 1997); cattle (Robl et al., 1986; Prather et al., 1987; Sims and First, 1993; U.S. Patents 4,994,384, 5,496,720, 5,057,420, 5,453,366); rabbits (Stice and Robl, 1988); pigs (Prather et al., 1989); mice (McGrath and Solter, 1983); and primates (Meng et al., 1997).

Cloning by nuclear transfer in mammals has several common steps. (For review see Prather and First, 1990, incorporated herein by reference.) First, a mature recipient oocyte arrested in Metaphase II is enucleated. Then a donor cell is placed under the zona pellucida of the recipient oocyte and the two are fused together to form a nuclear transfer embryo. Finally, it is necessary to activate the nuclear transfer embryo. Activation is most often accomplished by the same electric pulse which was used to fuse the donor cell and recipient oocyte, but may be also be accomplished by some other stimulus which increases the intracellular calcium concentration of the nuclear

transfer embryo.

For cows and sheep, it has traditionally been necessary to use aged recipient oocytes. The reason for using aged oocytes is that when younger oocytes are used as recipients the nuclear transfer embryo fails to activate. Activation is necessary for the new zygote or nuclear transfer embryo to exit Metaphase II arrest. While sperm are able to activate bovine oocytes after 24 hours of in vitro maturation, oocytes may be activated by stimuli which increase intracellular calcium concentration only after about 42 hours of in vitro culture. (Susko-Parrish et al., 1994). Recently, a method has been described by which bovine oocytes can be successfully activated after only 16 hours of in vitro culture. (Susko-Parish, U.S. Patent 5,496,720, incorporated herein by reference.) This two step method produces an increase in intracellular calcium concentration by treatment with the calcium ionophore inonomycin which is followed by the use of a serine-threonine kinase inhibitor to suppress cellular phosphorylation. It is hypothesized that the serine-threonine kinase inhibitor acts predominantly by inhibiting the c-Mos protein. c-Mos is thought to be necessary to maintain Metaphase II arrest, probably by maintaining the cell cycle protein p34cdc2 in an active state. p34cdc2 in turn is a protein kinase responsible for phosphorylating many proteins which are necessary for oocyte maturation and for normal mitotic cell division. Fertilization of aged oocytes generally results in lower rates of development than when younger oocytes are used. The ability to activate bovine oocytes at an earlier time after the beginning of in vitro culture means nuclear transfer embryos can be activated at a time correlating to the highest rates of development as determined by in vitro fertilization studies.

Traditionally, researchers have found that differentiated cells could not be used for nuclear

transfer because they would fail to support complete development. Studies in amphibians indicated that as more and more developmentally advanced nuclei are used for nuclear transfer, development decreases. (For review see Gurdon, 1986.) For this reason, many researchers concentrated on culturing embryonic stem cells (ES cells). ES cells are derived from undifferentiated germ cells or embryos and are cultured so as to maintain the cells in an undifferentiated, pluripotential state. ES cells were first described for the mouse and are used to create chimeric embryos since nuclear transfer does not work in mice. (For review see Stewart, 1991; Anderson, 1992.) Nuclear transfer embryos have been produced from ES cells in cattle (Sims and First, 1993) and sheep (Campbell et al., 1996, incorporated herein by reference). However, these methods are somewhat inefficient since only low numbers of the ES cells can be produced.

Recently, researchers have described a method for the production of nuclear transfer sheep embryos from a differentiated adult cell line and from cell lines derived from differentiated embryos (Wilmut et al., 1997, incorporated herein by reference). The donor cells are serum starved by culturing in low serum medium for five days before nuclear transfer. This causes the cells to arrest in G_0 The researchers hypothesize that nuclei arrested in G_0 are more amenable to nuclear remodeling and reprogramming. Nuclear remodeling and reprogramming describe the events whereby the donor cell nucleus is altered morphologically and biochemically to resemble a normal early embryo nucleus in morphology and transcriptional competence. (For review see Prather and First, 1990; Fulka et al., 1996.) In effect, the donor cell nucleus is de-differentiated.

This new nuclear transfer procedure may have several important impacts. First, genetically identical clones may be made of adult animals with

economically valuable traits such as high milk production. Second, the large numbers of donor cells which may be generated by normal cell culture techniques make possible the use of standard cell transfection techniques. For example, the donor cells may be transfected with exogenous DNA and a selectable marker. (For review see Joyner, 1991; Stewart, 1991.) Additionally, homologous recombination techniques can be used to add or delete genes from the donor cells at specific sites within the genome. (Koller et al., 1989; Cappecchi, 1989; Stanton et al., 1990, herein incorporated by reference.) After genetic modification of the donor cells by transfection with a selectable marker or homologous recombination, the donor cell can be used in the nuclear transfer procedure to produce a transgenic animal. The availability of these techniques should greatly increase the efficiency of producing transgenic animals other than mice. These animals may be made transgenic for economically valuable traits or for pharmaceutical proteins which may be harvested from the animals milk or blood. Third, this new technique of nuclear transfer may be used to propagate endangered species where it would be impossible to obtain enough gametes to optimize cloning procedures.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an efficient method of producing cloned embryos from differentiated cells by nuclear transfer. It is another object of this invention to provide a method for cloning donor cells of one species by utilizing a recipient oocyte from another species. It is still another object of this invention to provide cloned embryos. The cloned embryos may be implanted into recipient animals to produce offspring or used to originate totipotent or pluripotent cell lines for

therapeutic uses. It is also an object of the present invention to provide methods for efficient production of transgenic animals and animals in which genes have been "knocked out" by homologous recombination.

Therefore, the present invention provides a method of producing nuclear transfer bovine embryos from bovine somatic donor cells and bovine recipient oocytes or from somatic donor cells of one species and recipient oocytes of another species. The donor cells may be selected from the group consisting of embryonic derived cells, germ cells, somatic cells, and genetically modified cells. In this method, the donor cells are isolated and then cultured in low serum medium to induce the donor cells to undergo G_0 arrest. The donor cells are then fused to an enucleated recipient oocyte to create a nuclear transfer embryo. When enucleated bovine recipient oocytes are used, the oocytes are preferably selected from the group of bovine oocytes undergoing nuclear maturation within 16 hours of beginning in vitro culture. Fusion may preferably be accomplished by application of an electric pulse 16-24 hours after the beginning of in vitro culture. The nuclear transfer embryo must then be activated. Preferably, activation is accomplished by elevating intracellular calcium within the embryo by the use of ionomycin and then incubating the embryo with a serine threonine kinase inhibitor such as dimethylaminopurine (DMAP). The activation step is preferably performed about from 4 to 16 hours after fusion, and most preferably from about 4 to 8 hours after fusion. Alternatively, activation may be performed about 16 to 52 hours after the beginning of in vitro culture, most preferably about 20 to 32 hours after the beginning of in vitro culture.

The present invention also provides embryos produced by the above process and totipotent or pluripotent cell lines derived from those embryos. These embryos individually comprise either cytoplasm

derived from a bovine oocyte, bovine cytoplasm derived from a differentiated bovine cell, cell membrane derived from a bovine oocyte, cell membrane derived from a differentiated bovine cell and nuclei derived from a differentiated bovine cell or cytoplasm and cell membrane from one species and differentiated cytoplasm, differentiated cell membrane, and nuclei derived from a differentiated cell of another species.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Cloning mammals by nuclear transfer is generally comprised of the following steps. First, the recipient oocyte is enucleated. Second, the donor cell is placed in contact with the enucleated oocyte and the two are fused together to form a nuclear transfer embryo. Third, the nuclear transfer embryo is activated so that development can occur. The basic cloning steps for bovine embryos have been described in U.S. Patents 4,994,384, 5,496,720, 5,057,420, and 5,453,366, incorporated herein by reference. Cloning procedures for sheep have been described by Willadsen, 1986, Campbell et al., 1996, and Wilmut at al., 1997, incorporated herein by reference.

Recently, researchers in Scotland demonstrated that somatic cells and embryonically derived cells could be used as donor cells for nuclear transfer. (Wilmut et al., 1997; Campbell et al., 1996.) The subject matter of these publications also appears in PCT Publications WO 97/07669, WO 97/07668 and WO 96/07732, incorporated herein by reference. Prior to these results, it was thought impossible to clone mammals or fully developmentally competent amphibians by nuclear transfer from cells which have undergone differentiation. (DiBerardino, 1997.) The entire concept of the use of embryonic stem cells to produce embryos was based on keeping the stem cells in undifferentiated state. The use of stem cells for

cloning purposes is described in PCT Publications WO 95/17500, WO 95/16770 and WO 95/08625, herein incorporated by reference.

The use of somatic cells or other differentiated cells for nuclear transfer in mammals has many potential uses. First, the cells of a genetically valuable animal such as a cow with high milk production may be used to make genetically identical cows. Second, endangered species could be propagated. Third, transgenic animals could be produced from cell lines transfected with exogenous DNA. The high numbers of somatic cells available mean that well developed means of transfecting cells with exogenous DNA can be used such as the use of selectable markers to select transfected cells and homologous recombination. By using these methods researchers should be able to produce transgenic animals far more efficiently than by currently used techniques such as pronuclear injection.

One of the most important aspects of this invention is the wide variety of cells which may be used. The term "donor cell", as used herein, means any cell of any species which is used to provide a nucleus to an enucleated oocyte. Types of donor cells include, but are not limited to, somatic cells which are cells derived from adult tissue, embryonic derived cells which are differentiated cells harvested from any stage of embryo or fetus, germ cells which are cells which form or a product of the germline lineage, and genetically modified cells which are cells into which exogenous DNA has been introduced by any transfection technique.

The donor cells used in the present invention must be serum starved before use. (Wilmut et al., 1997; Campbell et al., 1996.) Serum starvation induces the donor cells to arrest in the G_0 stage of the cell cycle. Serum starvation is a simple technique whereby the normal concentration of serum in cell culture media, generally about 10 percent, is substantially

lowered for several days. For example, Wilmut et al., cultured the donor cells used in their experiments for 5 days in a medium with 0.5 percent fetal calf serum. The reason why this treatment produces cells which may be used as donor cells for nuclear transfer is unknown. One theory is that the serum starvation causes changes in chromatin structure which allows for reprogramming of the donor nucleus by the recipient oocyte. An additional benefit of serum starvation is that the cells uniformly arrest at a defined stage of the cell cycle, G_0. G_0 is a stage of quiescence in cell growth the cells enter into just after mitosis. Actively growing cells generally enter into G1 and prepare for DNA synthesis if not serum starved. By inducing the G_0 arrest, all the cells used for subsequent cloning are at the same stage of the cell cycle, in contrast to actively growing cells which are at various stages of the cell cycle. The advantage is that the G_0 arrested cells have a 2N amount of DNA which corresponds to the normal amount of DNA contributed to a newly fertilized egg by the oocyte and sperm. Therefore, the nuclear transfer embryos begin development with the correct amount of DNA and correct ploidy.

The term "low serum medium" as used herein means cell culture medium that is supplemented with a lower than normal amount of serum, such as fetal calf serum, so that cells cultured in the medium undergo G_0 arrest. "G_0 arrest" means that stage of the cell cycle after mitosis and before DNA synthesis where the cell is quiescent and ceases preparation for DNA synthesis and further cell division. The medium used may be any medium in which the donor cell type is normally cultured. Examples of such media include but are not limited to Ham's F 10, TCM-199, and DMEM. In the preferred embodiment of this invention, donor cells are cultured in low serum medium for a period of time sufficient to induce G_0 arrest. In the most preferred embodiment, the donor cells are cultured for five days

in alpha-MEM with no serum.

The next step in cloning by nuclear transfer involves the culture, selection and enucleation of the recipient oocyte. "Recipient oocyte" means a mature oocyte which is at the correct stage of development to be fertilized by a sperm cell and which after enucleation, serves as the recipient for the donor cell nucleus. For cloning procedures in most mammals, it is necessary to use mature oocytes which are arrested in Metaphase II. (Prather and First, 1990.) This is the stage at which fertilization leading to competent development takes place in most mammalian species. For species such as dogs in which fertilization takes place at a different stage of oocyte development, it would be necessary to utilize an oocyte of the appropriate stage. The use of Metaphase II oocytes also facilitates enucleation because the Metaphase II plate is located adjacent to the first polar body.

The recipient oocyte may be matured either in vitro or in vivo. In the preferred embodiment of the invention, the recipient oocyte is a mature oocyte of any species. In the most preferred embodiment, the recipient oocyte is a mature bovine oocyte. In vivo matured bovine oocytes may be recovered surgically from either superovulated or nonsuperovulated cows or heifers 35 to 48 hours past the onset of estrus or past an injection of human Chorionic Gonadotropin (hCG) or similar hormone. In vitro matured bovine oocytes may be prepared by the methods of Sirard et al., (1988) Parrish et al., (1988) and Rosenkrans and First (1993), incorporated herein by reference. Briefly, ovaries are collected at a slaughterhouse and transported to the laboratory in normal saline at 30 to 34° C. Transport varies from 2 to 6 hours. Oocytes from small follicles of 1 to 6 mm in diameter are aspirated. The immature oocytes are then washed three times in TL HEPES without glucose and to which 0.22 mM pyruvate and 1 mg/ml BSA have been added. The immature oocytes are then placed

in 50 μl drops of maturation medium under paraffin oil. Maturation medium is composed of TC199 containing 10 percent heat treated fetal calf serum, 0.22 mM pyruvate, 0.5 μg/ml FSH-P (Scherring-Plough Animal Health Corp. Kenilworth, N.J.) and 1 μg/ml estradiol. Ten oocytes are placed in each drop and incubated overnight at 39° C, in high humidity atmosphere comprised of 5% CO_2 and 95% air. The term "beginning of in vitro culture" means the time at which the recipient oocytes are placed in maturation medium.

In a further preferred embodiment, the recipient bovine oocytes are selected from the group of oocytes which mature within 16 hours of the beginning of in vitro culture. To select such oocytes at 16 hours, it is necessary to remove the enclosing cumulus cells from the oocytes so that the extrusion of the first polar body may be observed. Cumulus may be removed by placing the oocytes in TL-HEPES medium supplemented with 2.0 mg/ml of hyaluronidase and then gently aspirating the oocytes with a fire-polished pipette tip. (Sims and First, 1994.) Oocytes which have extruded the first polar body are in Metaphase II arrest and mature. The reason for selecting this subpopulation of oocytes is that they support a higher level of embryo development after fertilization as demonstrated by Dominko and First, 1997.

In the preferred embodiment, recipient oocytes are enucleated at an appropriate time after maturation has occurred, normally 1 to 15 hours after extrusion of the first polar body. In the most preferred embodiment, bovine oocytes are enucleated 16-20 hours after the beginning of in vitro culture. The procedure for enucleation is as follows and is described in Prather et al., U.S. Patent 4,994,384, incorporated herein by reference. Cumulus cells are removed from the oocytes by placing the oocytes in TL-HEPES medium supplemented with 2.0 mg/ml of hyaluronidase and then gently aspirating the oocytes with a fire-polished pipette

tip. (Sims and First, 1994.) Oocytes to be enucleated are then placed in culture dishes in 100 μl drops under oil of TL-HEPES medium supplemented with calcium and magnesium. (See generally Sims and First, 1994.) Micromanipulation may be performed with a Nikon Diaphot microscope equipped Hoffman optics and Narishige micromanipulators. The recipient oocyte is held with a holding pipet with a 120 μm outer diameter and 30 μm inner diameter. Enucleation is performed by using a beveled, sharpened enucleation pipet with an outer diameter of approximately 25 μm. The inner diameter may be varied according to the size of the donor cell which is used. To enucleate the oocyte, the enucleation pipet is inserted into the oocyte and a small amount of cytoplasm is removed just below the first polar body. Preferably, enucleation can be verified by staining the mature recipient oocyte with Hoechst 33342, visualizing DNA by excitation emission upon exposure to ultraviolet light for a short time (i.e. 5 seconds), enucleating the recipient oocyte, and washing the oocyte 2 times in TL-HEPES.

The next steps involve placing the donor cell under the zona pellucida of the recipient oocyte and fusing the recipient oocyte with donor cell. The donor cell is placed under the zona pellucidae of the recipient oocyte by use of the enucleation pipet. In the preferred embodiment the donor cell and the recipient oocyte may come from the same species or different species and are fused at the time appropriate for the recipient oocyte species. Examples include but are not limited to a bovine somatic cell with a bovine recipient oocyte, a sheep somatic cell with a bovine recipient oocyte, or a genetically altered or unaltered non-human primate cell or human cell with a bovine oocyte.

In the most preferred embodiment, a donor cell which has been cultured in low serum medium is fused at 16-24 hours after initiation of in vitro culture to a

recipient bovine oocyte selected from the group of oocytes maturing within 16 hours from the beginning of culture. Two main advantages exist for utilizing this method of cloning for bovine donor cells and bovine recipient oocytes. First, genetically valuable cows, such as those that exhibit high milk production or other valuable traits, can be multiplied from the various types of donor cells. Second, transgenic animals can be produced in an efficient manner. Standard methods of DNA transfection along with selectable markers can be utilized with large populations of donor cells. (For review see Joyner, 1991; Stewart, 1991.) Also, homologous recombination techniques can be used to introduce exogenous DNA or to knock out endogenous genes. (Koller et al., 1989; Cappecchi, 1989; Stanton et al., 1990.) The exogenous DNA introduced into the donor cells may be a gene encoding a valuable trait or a gene encoding a pharmaceutical protein which will be harvested from the animal.

These advantages are also applicable when the donor cells and recipient oocytes are derived from different species. In another most preferred embodiment, donor cells from species including but not limited to sheep, pigs, rats, non-human primates and humans are fused to the bovine recipient oocyte. An additional advantage of this method is that nuclear transfer clones of these species may be made utilizing the well-defined bovine recipient oocyte. Maturation procedures and timing for fusion and activation have been optimized in the bovine system. When bovine eggs are used, these two parameters do not have to be optimized as they would need to be if a recipient oocyte from another species is utilized. This may be of special importance in the cloning of endangered species where there are too few animals from which gametes can be obtained to conduct experiments to optimize maturation, culture conditions, and timing for

developmental competence.

Using the bovine oocyte as the recipient oocyte for donor cells of different species raises several interesting questions. Nuclear transfer utilizing donor cells and recipient oocytes from different species provides a powerful experimental system to analyze early embryo development and the common characteristics of early embryo development shared across species. First, it is known that embryos of different species undergo a period of maternally directed cleavage divisions. These cleavage divisions occur in the absence of transcription from the embryonic genome. The time at which the embryo begins transcription is termed the maternal to embryonic transition (MET). (For review see Telford et al., 1993.) For example, bovine embryos begin transcription at the late 4 to early 8 cell stage, murine embryos at the 2 cell stage, rat embryos at the 2 cell stage, sheep embryos at the 8 cell to 16 cell stage and primate embryos at the 4 cell stage. (Telford et al., 1993.) So the timing of MET is specific to each species. Likewise, the complement of proteins which is initially produced by the embryonic genome seems to vary among species where this has been studied. (Crosby et al., 1988; Barnes and First, 1991; Connover et al., 1991.)

Here, the inventors describe the surprising result that the bovine recipient oocyte is capable of supporting the MET of various species when donor cells of various species are fused to the bovine recipient oocyte to produce a nuclear transfer embryo. Factors present in the bovine recipient oocyte cytoplasm are therefore probably responsible for the MET and subsequent transcription and translation of the species characteristic MET proteins because the pre-MET embryo is transcriptionally incompetent. This is evidenced by the fact that the nuclear transfer embryos progress through the MET and that the first readily observable

signs of differentiation, compaction and blastocoel formation, occur as in normal embryos.

Furthermore, the bovine recipient oocyte is apparently capable of supporting nuclear remodeling in the donor cells of various species. Nuclear remodeling refers to the series of changes that occur to the nucleus of the donor cell after nuclear transfer. (For review see Fulka et al., 1996; Prather and First, 1990.) The morphological and biochemical changes in the nucleus are probably due to factors present in the cytoplasm of the recipient oocyte. These changes include the disappearance of differentiated nucleoli, swelling of the transferred nucleus, and in amphibians the exchange of proteins between the nucleus and cytoplasm. These changes are probably necessary to support further development. Again, the bovine recipient oocyte apparently has the necessary proteins to support this remodeling in other species.

Third, the bovine recipient oocyte is probably capable of supporting correct changes in methylation of the DNA of the donor cells of the various species. DNA methylation has been shown to be a mechanism for the regulation of gene expression in mammals. (For review see Brandeis et al., 1993.) The DNA of mouse gametes is heavily methylated. Upon fertilization the DNA of the newly formed zygote is demethylated. At gastrulation, a bimodal pattern of methylation is formed with CpG island containing genes being demethylated and most other genes being methylated. Tissue specific genes are demethylated as differentiation continues. The bovine recipient oocyte may be able to support the initial wave of demethylation as evidenced by the developmental competence of the nuclear transfer embryos. After the MET, the genome of the donor cell probably exerts itself to establish correct methylation patterns.

In the preferred embodiment, fusion of the recipient oocyte and donor nucleus is performed by

electric pulse. In the most preferred embodiment, the bovine recipient oocyte is fused to the bovine donor cells or donor cells of other species by a double electric pulse in sucrose or sorbitol fusion medium in a Zimmerman Electrofusion Instrument (CGA Corporation, Chicago, Illinois). The method is similar to that described in Prather et al., U.S. Patent 4,994,384. The Electrofusion Instrument may be adjusted as follows:

Fusion voltage: 90 volts (DC, 1.8 KV/cm);
Electrode distance: 0.5 millimeter;
Alignment voltage: 1-5 volts (AC);
Alignment frequency: 1000 KHZ;
Pulse duration: 30 microseconds, two pulses;
Postfusion alignment time: 5 seconds

Sorbitol fusion medium consists of 0.25 M d-sorbitol in medical grade H20 (Baxter), 100 μM Ca-acetate, 0.5 mM Mg-acetate, and 1 g/l Fatty Acid Free BSA (pH 7.2 at room temperature, 255 mOsm). Sucrose fusion medium consists of 95.84 g/l sucrose, 0.107 g/L Mg-acetate-4-H_2O, 0.016 ,μM Ca-acetate, 0.174 g/l KH_2PO_4 anhydrous, 0.031 g/l glutathione, and 0.01 g/l Fatty Acid Free BSA, ELISA grade (260-290 mOsm, pH 7.0). Other fusion media may be used such as Zimmerman Cell Fusion Medium, mannitol, and phosphate buffer solution.

Alternatively, fusion may be accomplished by treatment of the donor cell and recipient oocyte with polyethylene glycol as described in Sims and First, 1994. Fusion may be accomplished also by use of Sendai virus. (Graham, 1969.)

The next step in cloning by nuclear transfer is activation of the nuclear transfer embryo. "Nuclear transfer embryo" means the embryo which results from the fusion of the donor cell and recipient oocyte. Normally, the oocyte is activated by the sperm when fertilized. The interaction of the sperm with the

oocyte cell membrane causes an elevation of intracellular calcium which starts a cascade of events including the release of the new zygote from Metaphase II arrest. Activation by fertilization is followed by pronuclear formation. Since nuclear transfer embryos are not fertilized, an activation stimulus is needed to release the nuclear transfer embryo from Metaphase II arrest. In traditional nuclear transfer protocols, the electric pulse used to fuse the donor cell and recipient oocyte acts as the activation stimulus. (Willadsen, 1986; Robl et al., 1986; Prather et al., 1987; Campbell et al., 1996.) It is necessary that sheep recipient oocytes and bovine recipient oocytes be aged in order for an electric pulse to be sufficient to activate the resulting nuclear transfer embryo. (Prather et al., 1987; Campbell et al., 1996.)

In a preferred embodiment, the nuclear transfer embryo is activated by a stimulus sufficient to release the nuclear transfer embryo from Metaphase II arrest. "Activate", as used herein, means the use of a stimulus sufficient to release the nuclear transfer embryo from Metaphase II arrest. In an alternative embodiment, a bovine nuclear transfer embryo is activated by the fusion electric pulse described above at 35 to 52 hours after the beginning of in vitro culture.

Recently, a method has been described for activating bovine oocytes as early as 10 hours after the beginning of in vitro culture. (Susko-Parish et al., U.S. Patent 5,496,720, incorporated herein by reference.) This method comprises the sequential incubation of an oocyte or nuclear transfer embryo with a calcium ionophore such as ionomycin, and a serine-threonine kinase inhibitor such as 6-dimethylaminopurine (DMAP). Treatment with ionomycin causes an increase in intracellular calcium similar to that normally caused by the sperm at fertilization. The role of DMAP is less clear, but it may act by inhibiting the serine-threonine kinase c-Mos which is

thought to stabilize the activity of p34cdc2 which is activated by phosphorylation on serine and threonine residues and inactivated by phosphorylation on tyrosine residues. High p34cdc2 activity is generally associated with Metaphase II arrest. Since the activity of c-Mos is inhibited, p34cdc2 activity is down regulated via the absence of an entity able to maintain the activating phosphorylations on p34cdc2's serine and threonine residues. One reason that aged oocytes may be activated solely by electrical pulse is that p34cdc2 activity in these cells has declined. All that is needed to activate aged oocytes is the elevation of intracellular calcium by one of several methods. To overcome the high levels of p34cdc2 activity in younger oocytes, both elevation of intracellular calcium and inhibition of serine-threonine kinases appear to be required.

In the preferred embodiment, nuclear transfer embryos are activated at an appropriate time (preferably about 4 to 16 hours after fusion) by increasing the concentration of intracellular calcium and incubation with a serine-threonine kinase inhibitor. In the most preferred embodiment, nuclear transfer embryos comprised of fused bovine recipient oocytes and bovine donor cells or donor cells from other species are activated by sequential incubation with ionomycin and DMAP at about 28 hours after the beginning of in vitro culture. Briefly, the nuclear transfer embryos are exposed to 5 μM ionomycin (5 mM stock in DMSO; Calbiochem, La Jolla, CA) in TL-HEPES (1 mg/ml fatty acid free BSA) for 4 minutes. The nuclear transfer embryos are then diluted into TL-HEPES (30 mg/ml fatty acid free BSA) for five minutes and then further diluted into TL-HEPES (1 mg/ml Fraction V BSA). The nuclear transfer embryos are then moved to 50 μl drops under paraffin oil of CRlaa with 1.9 mM DMAP and incubated for 4 hours at 39° C, 5% CO_2 in air, high humidity. After this incubation, embryos are diluted

in TL HEPES with 1 mg/ml BSA and then transferred into CRlaa media and cultured at 39° C, 5% CO_2 in air, high humidity. Other methods which may be used to increase intracellular calcium include ethanol treatment, A23187, electric shock and caged chelators. Other serine-threonine kinase inhibitors which may be used include staurosporine, 2-aminopurine, and sphingosine. For a further description of these methods see Susko-Parrish et al., U.S. Patent 5,496,720, incorporated herein by reference.

There are two main advantages to the use of the ionomycin/DMAP activation regimen. First, the nuclear transfer embryo may be activated at the time when developmental competence is highest as demonstrated by studies of the fertilization of bovine oocytes. Traditional cloning techniques require that the recipient oocyte be aged before fusion and activation. The use of aged oocytes may compromise the development of the resulting nuclear transfer embryo. Second, p34cdc2 has been shown to have a positive effect on nuclear remodeling. (Fulka et al., 1996.) The nuclei of nuclear transfer embryos which are exposed to a cellular environment with high levels of p34cdc2 activity remodel more completely and more quickly than when fusion occurs to recipients that have already been preactivated or which have low levels of p34cdc2 activity. One reason that aged oocytes may be activated solely by electrical pulse is that p34cdc2 activity in these cells has declined. Since p34cdc2 activity has declined in aged recipient oocytes, nuclear remodeling may not be complete. With the ionomycin/DMAP regimen, at the time of fusion, which occurs prior to activation, the donor cell nucleus is exposed to high levels of p34cdc2 activity which is beneficial to nuclear remodeling.

Temporal separation of the fusion and activation steps allows the nuclear transfer embryo sufficient time to remodel its nucleus. In the most preferred

embodiment, fusion occurs at 16 hours after the beginning of in vitro culture while activation occurs 28 hours after the beginning of in vitro culture. This procedure differs from previously described nuclear transfer techniques where fusion and activation are simultaneous. The examples verify that this method results in an approximately 2 fold increase to blastocyst than previously described somatic cell cloning methods.

After activation of the nuclear transfer embryo, it is necessary to either culture the embryo in vitro or transfer the embryo to an appropriate recipient animal. In the preferred embodiment, nuclear transfer embryos comprised of bovine recipient oocytes and bovine donor cells are cultured until the blastocyst stage in vitro and then transferred to a recipient. In vitro culture is carried out as described in Sims and First, 1994 and Rosenkrans et al., 1993. Briefly, nuclear transfer embryos may be cultured in a defined medium, CRlaa or CR2 for 7-8 days at 39° C in 5% CO_2/95% air in high humidity. CRlaa is comprised of 5 mM hemicalcium lactate, 114.7 mM sodium chloride, 3.1 mM potassium chloride, 26.2 mM sodium bicarbonate, and 3 mg/ml fatty acid free bovine serum albumin. (Rosenkrans et al., 1993.)

EXAMPLES

Methods Common to all Experiments

Oocyte maturation- In vitro matured bovine oocytes may be prepared by the methods of Sirard et al. (1988), Parrish et al. (1988) and Rosenkrans and First (1993). Briefly, ovaries are collected at a slaughterhouse and transported to the laboratory in normal saline at 30° to 34° C. Transport time varies from 2 to 6 hours. Oocytes from small follicles of 1 to 6 mm in diameter are aspirated. The immature oocytes are then washed

three times in TL-HEPES without glucose and to which 0.22 mM pyruvate and 1 mg/ml BSA have been added. The immature oocytes are then placed in 50 μl drops of maturation medium under paraffin oil. Maturation medium is composed of TC199 containing 10 percent heat treated fetal calf serum, 0.22 mM pyruvate, 0.5 μg/ml FSH-P (Scherring-Plough Animal Health Corp. Kenilworth, N.J.) and 1 μg/ml estradiol. Ten oocytes are placed in each drop and incubated overnight at 39° C, in high humidity atmosphere comprised of 5% CO_2 and 95% air.

Micromanipulation- Enucleation was performed at 16-20 hours after the beginning of in vitro culture. The procedure for enucleation is as follows and is described in Prather et al., U.S. Patent 4,994,384, incorporated herein by reference. Cumulus cells are removed from the oocytes by placing the oocytes in TL-HEPES medium supplemented with 2.0 milligrams per milliliter of hyaluronidase and then gently aspirating the oocytes with a fire-polished pipette tip. (Sims and First, 1994.) Oocytes to be enucleated are then placed in culture dishes in 100 microliter drops under oil of TL-HEPES medium supplemented with calcium and magnesium. (See generally Sims and First, 1994.) Micromanipulation may be performed with a Nikon Diaphot microscope equipped Hoffman optics and Narishige micromanipulators. The recipient oocyte is held with a holding pipet with a 120 μm outer diameter and 30 μm inner diameter. Enucleation is performed by using a beveled, sharpened enucleation pipet with an outer diameter of approximately 25 μm. The inner diameter may be varied according to the size of the donor cell which is used. To enucleate the oocyte, the enucleation pipet is inserted into the oocyte and a small amount of cytoplasm is removed just below the first polar body. Preferably, enucleation can be verified by staining the mature recipient oocyte with Hoechst 33342, visualizing DNA by excitation emission

upon exposure to ultraviolet light for a short time (i.e. 5 seconds), enucleating the recipient oocyte, and washing the oocyte 2 times in TL-HEPES. The donor cell is placed under the zona pellucidae of the recipient oocyte by use of the enucleation pipet.

Fusion- Fusion of the bovine, non-human primate, rat, sheep and porcine donor cells to the bovine recipient oocytes was performed at 16-24 hours after the beginning of in vitro culture. The bovine recipient oocyte is fused to the bovine donor cells or donor cells of other species by a double electric Zimmerman Electrofusion Instrument (CGA Corporation, Chicago, Illinois). The method is similar to that described in Prather et al., U.S. Patent 4,994,384. The Electrofusion Instrument was adjusted as follows:

Fusion voltage: 90 volts (DC, 1.8 KV/cm);
Electrode distance: 0.5 millimeter;
Alignment voltage: 1-5 volts (AC);
Alignment frequency: 1000 KHZ;
Pulse duration: 30 μseconds, two pulses;
Postfusion alignment time: 5 seconds

Two types of fusion media were utilized. The first consisted of 0.25 M d-sorbitol in medical grade H_20 (Baxter), 100 μM Ca-acetate, 0.5 mM Mg-acetate, and 1 g/1 Fatty Acid Free BSA (pH 7.2 at room temperature, 255 mOsm). The second consisted of 95.84 g/1 sucrose, 0.107 g/L Mg-acetate-4-H_2O, 0.016 μM Ca-acetate, 0.174 gA KH_2PO_4 anhydrous, 0.031 g/1 glutathione, and 0.01 g/1 Fatty Acid Free BSA, ELISA grade (260-290 mOsm, pH 7.0).

Cell culture- Somatic cells were obtained from bovine, sheep, non-human primate (Rhesus), porcine and rat ear skin samples, removed from live animals. The skin samples were placed into PBS (phosphate buffered

saline, pH) supplemented with antibiotic/antimycotic (Gibco), and washed 3 times. All hair was manually removed and the sample cut into small pieces. The cut samples were then placed in 0.05% trypsin/1 mM EDTA solution (Gibco) for 40 minutes at 30° C and stirred frequently during incubation. The samples were then centrifuged (10 minutes, 1000 rpm) and supernatant containing single cells removed to remove undigested pieces of tissue. The final supernatant was placed into alpha-MEM (Gibco), supplemented 10% fetal calf serum (HiClone) and antibiotic/antimycotic (Gibco) and cultured in tissue flasks (Falcon, 25 cm^2) at 37° C for at least a week. After a monolayer of cells had formed, the medium was removed and the monolayer washed with Ca-Mg free PBS once and replaced by trypsin/EDTA solution and incubated at 37° C for 2-3 minutes. Cells are then desegregated by pipetting and centrifuged as described above. The pellet of cells was diluted 1:2 with alpha-MEM and placed into two flasks. All subsequent passages were performed in the same manner every 2 days thereafter.

For nuclear transfer, the cells were removed from flasks as described for passaging and placed into a 35 mm Petri dish (Falcon tissue culture) and grown in alpha-MEM for 2-12 days in the absence of any serum. On the day of nuclear transfer, the cells grown in the absence of sera were trypsinized as described above, washed in Ca-Mg free PBS and placed into TL HEPES supplemented with 0.1 -3 mg/ml BSA Faction V, 0.2 mM Na-pyruvate and 25 μg/ml gentamicin.

Activation of nuclear transfer embryos- Activation was performed at 24-28 hours after the beginning of in vitro culture. The nuclear transfer embryos were exposed to 5 μM ionomycin (5 mM stock in DMSO; Calbiochem, La Jolla, CA) in TL-HEPES (1 mg/ml fatty acid free BSA) for 4 minutes. The nuclear transfer embryos were then diluted into TL-HEPES (30 mg/ml fatty

acid free BSA) for five minutes and then twice diluted into TL-HEPES (3 mg/ml Fraction V BSA). The nuclear transfer embryos were then moved to 50 microliter drops under paraffin oil of CR1aa supplemented with 1.9 mM DMAP and incubated for 4 hours at 39° C, 5% CO_2 in air, high humidity.

Culture after activation- After removal from DMAP, nuclear transfer embryos were diluted twice in TL HEPES supplemented with 3 mg/ml BSA Fraction V. All embryos, regardless of species, were cultured in CR1aa. CR1aa is comprised of 5 mM hemicalcium lactate, 114.7 mM sodium chloride, 3.1 mM potassium chloride, 26.2 mM sodium bicarbonate, and 3 mg/ml fatty acid free bovine serum albumin. (Rosenkrans et al., 1993.) Incubations were conducted at 39° C, 5% CO_2 in air, high humidity. After three days of culture, 10% heat treated FCS was added to all embryos, regardless of species and the embryos allowed to develop to blastocyst.

Results

Nuclear transfer embryos were constructed from bovine recipient oocytes and the following cell lines: bovine adult fibroblasts (BAF), bovine fetal fibroblasts (BFF), sheep adult fibroblasts (SAF), porcine adult fibroblasts (PAF), non-human primate adult fibroblasts (MAF) and rat adult fibroblasts (RAF). The results are summarized in Table 1.

Table 1: Ability of donor cells to initiate embryonic development after nuclear transfer into a recipient bovine oocyte.

Donor Cell Line	Nuclear Transfer (N)	Fused (N)	Lysed or not fused(N)	CLV.* (%/fused)	CM+BL** (%/C.V.)
BAF	220	145	75	81/145	22/81
BFF	82	51	31	31/51	18/31
SAF	327	182	135	120/135	38/120
PAF	93	36	57	32/57	12/32
RAF	108	33	75	37/	na
MAF	30	28	2	24/28	14/24

* CLV. - number of embryos undergoing cleavage
** CM-BL- number of embryos developing to compact morula or blastocyst

Column 1 indicates the donor cell line, column 2, Nuclear Transfer, indicates the number of nuclear transfers attempted, column 3, Fused, indicates the number of fused nuclear transfer embryos produced, column 4, Lysed, indicates the number of recipient oocytes which Lysed or where fusion did not occur, column 5, CLV, indicates the number nuclear transfer embryos which cleaved and is expressed as a percentage of fused nuclear transfer embryos, column 6, CM+BL, indicates the number of nuclear transfer embryos developing to compacted morula or blastocyst and is expressed as a percentage of cleaved embryos.

These results indicate the bovine oocyte can be utilized as the recipient oocyte in combination differentiated donor cells from cattle, pigs, monkeys, rats and sheep to create nuclear transfer embryos. The methods employed lead to a greater efficiency of development to compacted morula and blastocyst than previously described somatic cell cloning procedures. Wilmut et al., reported the development of 29 embryos

to compacted morula or blastocyst out of 277 fused nuclear transfer embryos (11.7%) when using somatic sheep cells. Here the researchers report the development of 38 compacted morulae or blastocysts out of 182 fused nuclear transfer embryos (20.9%), An increase in efficiency of about two fold.

To date, 13 embryos derived from bovine adult fibroblasts have been transferred to recipient cows, 8 embryos derived from sheep adult fibroblasts have been transferred to sheep, and 20 embryos derived from rat adult fibroblasts have been transferred to rats.

REFERENCES

Anderson, *Animal Biotechnology* 3, 165-175 (1992).

Barnes and First, Embryonic Transcription in in vitro Cultured Covine Embryos, *Molecular Reproduction and Development* 29, 117-123 (1991).

Brandeis et al., Dynamics of DNA Methylation During Development, *BioEssays*, 15 709-713 (1993)

Campbell et al., Sheep Cloned by Nuclear Transfer from a Cultured Cell Line, *Nature*, 380 64-66 (1996)

Capecchi, Altering the Genome by Homologous Recombination, *Science* 244, 1288-1292 (1989).

Connover et al., Stage Specific Expression of a Family of Proteins that are Major Products of Gene Activation in the Mouse Embryo, *Developmental Biology* 147, 403-414 (1991).

Crosby et al., Control of Protein Synthesis During Early Cleavage of Sheep Embryos, *Journal of Reproduction and Fertility* 82, 769-775 (1988).

DiBerardino, Genornic Potential of Differentiated Cells Analyzed by Nuclear Transplantation American Zoology, 27, 623-644 (1987).

Dominko and First, Timing of Meiotic Progression in Bovine Oocytes and Its Effect on Early Embryo Development, *Molecular Reproduction and Development*, 47, 456-467 (1997).

First et al., Systems for Production of Calves from Cultured Bovine Embryonic Cells, *Reproduction, Fertility and Development*, 6 553-562 (1994).

Fulka et al., Nuclear Transplantation in Mammals: Remodeling of Transplanted Nuclei Under the Influence of Maturation Promoting Factor, *BioEssays*, 18 835-840 (1996).

Graham, The Fusion of Cells with One and Two Cell Mouse Embryos, *Wistar. Inot. Symp. Monogr.*, 9, 19 (1969).

Gurdon, Nuclear Transplantation in Eggs and Oocytes, *Journal of Cell Science* 4 (Supp.) 287-318 (1987)

Joyner, Gene Targeting and Gene Trap Screens Using Embryonic Stem Cells: New approaches to Mammalian Development, *BioEssays* 13, 649-658 (1991).

Koller et al., Proceedings of the National Academy of Sciences, USA 86, 8927-8931 (1989).

McGrath and Solter, Nuclear Transplantation in the Mouse Embryo by Microsurgery and Cell Fusion, *Science* 220, 1300-1302 (1983).

Meng et al., Full Term Development of Rhesus Monkeys Produced by Nuclear Transfer, *Biology of Reproduction* (Supp.) 56, 87 (1997).

Parrish et al., Capacitation of Bovine Sperm by Heparin, *Biology of Reproduction* 38, 1171-1180 (1988)

Prather et al., Nuclear Transplantation in Early Pig Embryos, *Biology of Reproduction*, 41 414 418 (1989).

Prather et al., Nuclear Lamin Antigens: Porcine and Bovine Early Developmental Regulation, *Biology of Reproduction*, 41 414-418 (1989).

Prather and First, Cloning Embryos by Nuclear Transfer, *Journal of Reproduction and Fertility*, Supplement 41, 125-134 (1990).

Robl et al., Nuclear Transplantation in Bovine Embryos, *Journal of Animal Science*, 64 642-647 (1986)

Robl et al., Nuclear Transplantation in the Bovine

Embryo: Assessment of Donor Nuclei and Recipient Oocyte, *Biology of Reproduction*, 37 859-866 (1987).

Rosenkrans et al., Journal of Animal Science 72, 634-637 (1993).

Sims and First, Production of Calves by Transfer of Nuclei from Cultured Inner Cell Mass Cells, *Proceedings of the National Academy of Sciences USA, 90* 6143-6147 (1993).

Sirard et al., The Culture of Bovine Oocytes to Obtain Developmentally Competent Embryos, *Biology of Reproduction 39*, 546-552 (1988).

Stanton et al., Germ Line Transmission of an Inactive N-myc Allele Generated by Homologous Recombination in Mouse Embryo Stem Cells, *Molecular and Cellular Biology* 10, 6755-6758 (1990)

Stewart, in Animal applications of Research in *Mammalian Development*, eds. Pederson, R. A., McLaren, A., and First, N. (Cold Spring Harbor Laboratory Press, Plainview, NY) 267-283 (1991).

Stice and Robl, Nuclear Reprogramming in Nuclear Transplant Rabbit Embryos, Biology of Reproduction 39, 657-664 (1988).

Susko-Parish et al., Inhibition of Protein Kinases after an Induced Calcium Transient Causes Transition of Bovine Oocytes to Embryonic Cycles without Meiotic Completion, *Developmental Biology* 166, 729-739 (1994).

Telford et al., Transition from Maternal to Embryonic Control in Early Mammalian Development: A Comparison of Species, *Molecular Reproduction and Development* 26, 90-100 (1990).

Willadsen, Nuclear Transplantation in Sheep Embryos, *Nature*, 320 63-65 (1986).

Wilmut et al, Viable Offspring Derived from Fetal and Adult Mammalian Cells, *Nature*, 385 810813 (1997).

Article 34

CLAIMS

What is claimed is:

1. A method of producing nuclear transfer embryos from donor cells of one species and recipient oocytes from another species comprising:

inducing the donor cells to undergo G_0 arrest;

fusing said donor cell to an enucleated recipient oocyte of another species to create a nuclear transfer embryo;

and activating said nuclear transfer embryo.

2. The method of claim 1 wherein said G_0 arrest of donor cells is induced by culture in low serum medium.

3. The method of claim 1 wherein said donor cells are selected from the group consisting of embryonic derived cells, germ cells, somatic cells, and genetically modified cells.

4. The method of claim 1 wherein said enucleated recipient oocyte is an enucleated bovine recipient oocyte.

5. The method of claim 4 wherein said enucleated bovine recipient oocyte is selected from the group of bovine oocytes undergoing nuclear maturation within 16 hours of beginning in vitro culture.

6. The method of claim 1 wherein said enucleated bovine recipient oocyte and said donor cell are fused by electric pulse to form a nuclear transfer embryo.

7. The method of claim 1 wherein said fusion is performed 16-32 hours after the beginning of in vitro culture.

8. The method of claim 1 wherein said nuclear transfer embryo is activated by elevating intracellular calcium and the incubating with a serine threonine kinase inhibitor.

9. The method of claim 8 wherein intracellular calcium is elevated by incubation with ionomycin and the serine threonine kinase inhibitor is DMAP.

10. The method of claim 1 wherein said activation is 16-32 hours after the beginning of in vitro culture.

11. The method of claim 6 wherein said fusion is 16-52 hours after the beginning of in vitro culture.

12. An embryo produced by the method of claim 1.

13. A method of producing nuclear transfer embryos from a donor cell of one species and a bovine recipient oocyte comprising:

culturing non-bovine donor cells selected from the group consisting of embryonic derived cells, somatic cells, germ cells, and genetically modified cells in low serum medium so that said donor cells are induced to arrest in the G_0 stage of the cell cycle;

selecting a bovine recipient oocyte which has completed nuclear maturation before 16 hours from the beginning of in vitro culture;

enucleating said bovine recipient oocyte after 16-32 hours of in vitro culture;

placing said donor cell under the zone pellucida of said enucleated oocyte so that said donor cell contacts said enucleated oocyte;

fusing said donor cell with said enucleated oocyte by electric pulse at 16-32 hours after the beginning of in vitro culture to create a nuclear transfer embryo;

and activating said nuclear transfer embryo by sequential incubation with ionomycin and 6-dimethylaminopurine at 16 to 32 hours after the beginning of in vitro culture.

14. The embryo produced by the process of claim 13.

15. A nuclear transfer embryo comprising cytoplasm and cell membrane from one species and differentiated cytoplasm, differentiated cell membrane, and nuclei derived from a differentiated cell of another species.

DECLARATION—Utility or Design Patent Application

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
	PCT/US98/15387	July 24, 1998	

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

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DECLARATION

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City	~~Oregon~~ BEAVERTON	State	~~WI~~ OR	Zip Code	~~52575~~ 97007 T.D. 4/29/2000	Country	US

Name of Additional Joint Investor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Maissam	Mitalipous

Inventor's Signature				Date			
Residence (City)	Madison	State	WI	Country	US	Citizenship	US
Post Office Address	2130 University Avenue #6						
Post Office Address							
City	Madison	State	WI	Zip Code	53705	Country	US

Name of Additional Joint Investor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname

Inventor's Signature				Date			
Residence (City)		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip Code		Country	

Name of Additional Joint Investor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname

Inventor's Signature				Date			
Residence (City)		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip Code		Country	

DECLARATION	Additional Inventor(s) Supplemental Sheet Page 1 of 1

Name of Additional Joint Investor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])					Family Name or Surname		
Tanja					Dominko		
Inventor's Signature				Date			
Residence (City)	Oregon	State	WI	Country	US	Citizenship	SL
Post Office Address	2320 White Oak Trail						
Post Office Address							
City	Oregon	State	WI	Zip Code	53575	Country	US

Name of Additional Joint Investor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])					Family Name or Surname		
Maissam					VA Mitalipov VA		
Inventor's Signature	5/8/00			Date	5/8/00		
Residence (City)	Madison	State	WI	Country	US	Citizenship	US
Post Office Address	2130 University Avenue #6						
Post Office Address							
City	Madison	State	WI	Zip Code	53705	Country	US

Name of Additional Joint Investor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])					Family Name or Surname		
Inventor's Signature				Date			
Residence (City)		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip Code		Country	

Name of Additional Joint Investor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])					Family Name or Surname		
Inventor's Signature				Date			
Residence (City)		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		Zip Code		Country	

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

Attorney Docket No.: 96429/9085

First Named Inventor: First et al.

COMPLETE IF KNOWN

Application Number: 09/463,276
Filing Date:
Group Art Unit:
Examiner Name:

☐ Declaration Submitted with Initial Filing

☒ Declaration Submitted after Initial Filing

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TRANS-SPECIES NUCLEAR TRANSFER

the specification of which

☐ is attached hereto

☒ was filed on as United States Application Number 60/053,103 filed July 26, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/053,103	26 July 1997